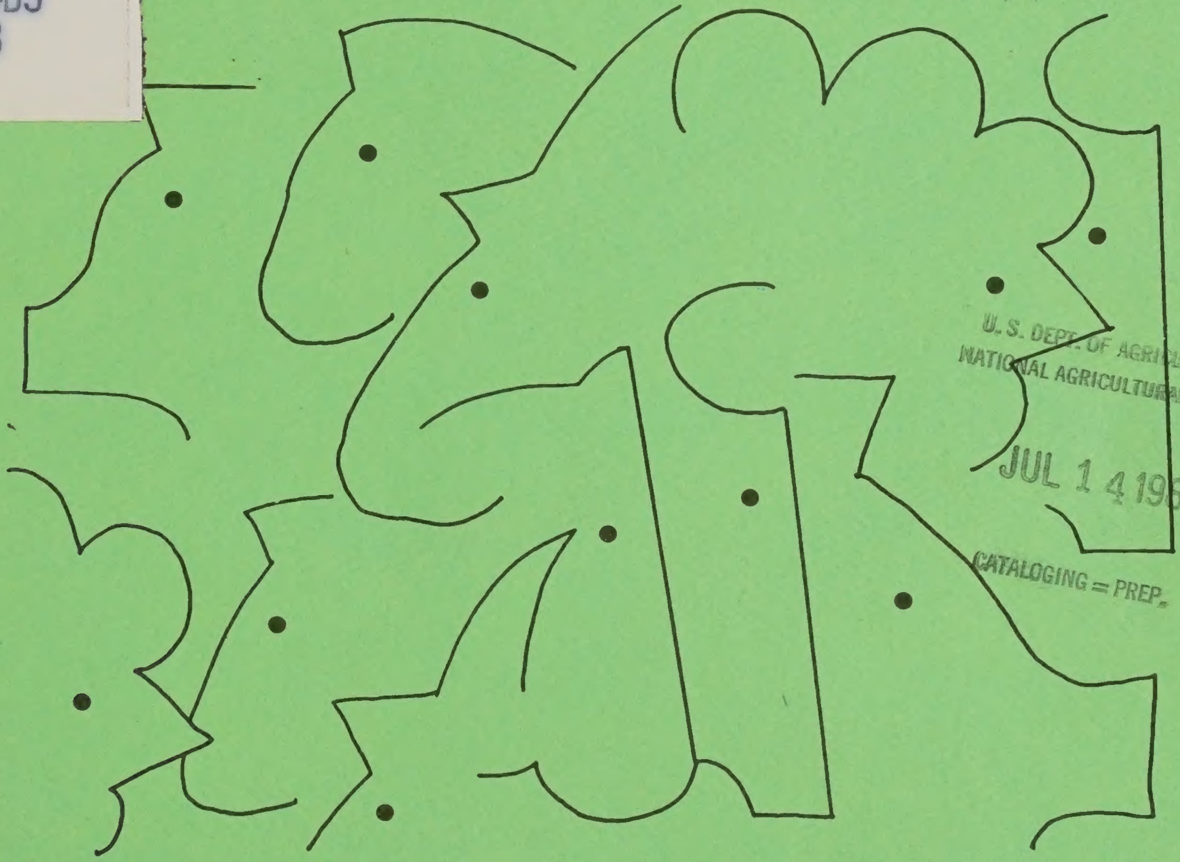
Diagnosis of African Swine Fever

[v.3]

IMMUNOELECTROOSMOPHORESIS - IEOP
AGAR GEL DIFFUSION PRECIPITATION - AGDP
TESTS

PLUM ISLAND ANIMAL DISEASE CENTER

E19722



UNITED STATES DEPARTMENT OF AGRICULTURE
SCIENCE AND EDUCATION ADMINISTRATION

FEDERAL RESEARCH
NORTHEASTERN REGION

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Introduction

Control and eradication of African swine fever (ASF) depend upon detection and elimination of exposed animals. The hemadsorption (HAd) test*, as well as immunofluorescence are valuable in the diagnosis of acute ASF. Where the disease has become enzootic in domestic swine, subacute and chronic infections are often encountered. Chronic infections stimulate complement fixing (CF) and AGDP antibodies against ASF virus, but detection by their use requires at least 12 hours. The IEOP test is more rapid and sensitive than either the CF or AGDP tests in detecting antibodies against ASF virus. (Pan, I. C., De Boer, C. J., and W. R. Hess.

*Please see Microfiche PIL-M-1.

African Swine Fever: Application of Immunoelectroosmophoresis for Detection of Antibody. Canadian Journal of Comparative Medicine, 36:309-316, 1972.)

Since it is seldom possible to establish a diagnosis of ASF by clinical means alone, laboratory tests are required to differentiate the disease from hog cholera and other diseases. This microfiche describes in detail how the IEOP, AGDP and FA tests for ASF are performed. Laboratory personnel with experience in cell culture (CC) should be able to carry out the diagnostic procedures described.

THE IEOP TEST

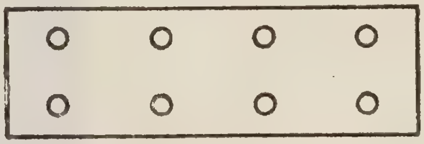
The next frame illustrates graphically the basic steps in the IEOP test. The test is fundamentally simple and is easily performed if the laboratory has good ASF viral antigen and an immunoelectrophoresis apparatus. Microscopic slides are coated with agar in which wells are made. Antigen is placed in wells nearest the cathode, or negative pole and serum in the opposite wells. Direct electric current is applied for about 30 minutes. At the end of that period the test may be read.

BASIC STEPS IN THE IMMUNOELECTROOSMOPHORESIS (IEOP) TEST FOR AFRICAN SWINE FEVER (ASF) ANTIBODIES

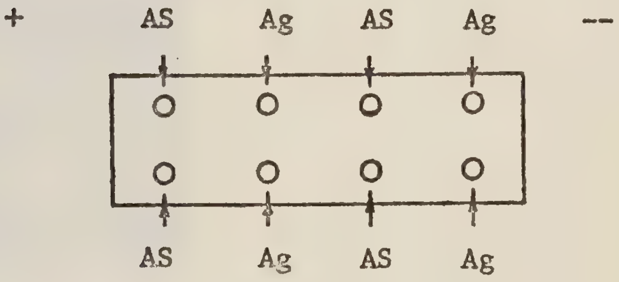
1. Ordinary microscope slides are coated with agar gel.

2. Wells are made in the agar.

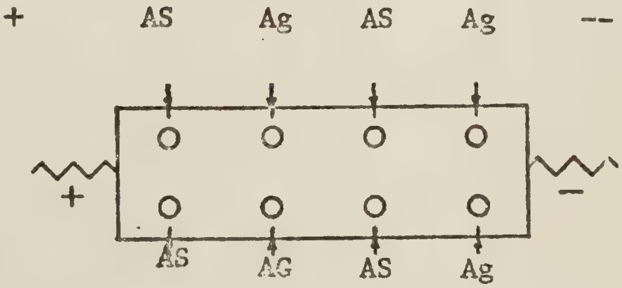
Diameter of well= 2.5mm
Interwell distance (center to center) = 13mm



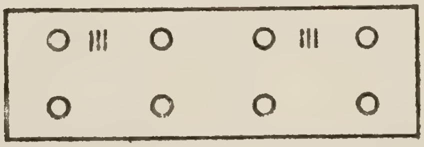
3. Known ASF antigen (AG) is placed in one of a pair of wells and serum(s) from a suspect animal in the other. (Control antisera are also used.)



4. Direct current is applied to the slide with the negative (-, cathode) pole connected to the side with antigen for 30 minutes.



5. Where the serum is positive, lines appear between the wells.



(Advantages: More than 100 tests can be performed at once. The test is accurate and sensitive requiring small amounts of reagents.)

POSITIVE IEOP TESTS

Wells on the left were filled with antisera (Lisbon" , "Rome") from swine infected with ASF virus. Wells on the right were filled with ASF viral antigen. Lines in the agar between the wells indicate positive sera.

TABLE I. The Sensitivity of the IEOP Test by Block Titration of Antigen and Antibody

Ag / As	u[a]	1:2	1:4	1:8	1:16	1:32	1:64	1:128
u	+	+	+	+	+	−	−	−
1:2	+	+	+	+	+	+	−	−
1:4	+	+	+	+	+	+	−	−
1:8	+	+	+	+	+	+	−	−
1:16	+	+	+	+	+	+	+	−
1:32	+	+	+	+	+	+	+	−
1:64	+	+	+	+	+	+	−	−
1:128	+	+	+	+	+	+	−	−
1:256	−	+	+	+	+	+	−	−
1:512	−	−	+	+	+	+	−	−
1:1024	−	−	−	+	+	−	−	−
1:2048	−	−	−	−	−	−	−	−

Note: Reagents volume = 5 μl. Final results recorded after staining

[a] u = undiluted

TABLE II. The Sensitivity of the CF Test[a] by Block Titration of Antigen and Antibody.

Ag / As	1:8	1:16	1:32	1:64	1:128	1:256
1:8	+	+	+	+	−	−
1:16	+	+	+	+	+	−
1:32	+	+	+	+	+	−
1:64	+	+	+	+	−	−
1:128	+	+	+	−	−	−
1:256	+	+	+	−	−	−
1:512	+	+	−	−	−	−
1:1024	−	−	−	−	−	−
1:2048	−	−	−	−	−	−

[a] The reaction mixtures contained 0.25 ml of serum dilution, 1.75 hemolytic units of guinea pig complement in 0.5 ml and 0.25 ml antigen dilution. Fixation was overnight at 5°C. Sensitized sheep red blood cells (2%) (0.5 ml) were added and the mixtures incubated for one hour at 37°C. Tubes which showed complete inhibition of hemolysis were expressed as +

TABLE III. The Sensitivity of the AGDP Test[a] by Block Titration of Antigen and Antibody

Ag / As	Undiluted	1:2	1:4	1:8
Undiluted	+	+	+	−
1:2	+	+	+	−
1:4	+	+	+	−
1:8	+	+	+	−
1:16	+	+	−	−
1:32	+	+	−	−
1:64	+	+	−	−
1:128	+	+	−	−
1:256	+	+	−	−
1:512	−	−	−	−

[a] Reagent volume = 30 µl. Well size was 4 mm in diameter, and the distance of antigen and antiserum was 4 mm. Results recorded five days incubation at room temperature.

TABLE IV. Detection of Antibody in Pig Sera by Three Different Tests

Infected With	IEOP			CF	AGDP	IEOP (+)[b] AGDP (+) CF (−)
	Undiluted[a]	Diluted	Staining			
Lisbon	115/120[c] (95.8%)	119/120 (99.1%)	120/120 (100%)	85/120 (70.8%)	84/120 (70.0%)	5/120 (4.1%)
Tengani	95/95 (100%)	95/95 (100%)	95/95 (100%)	33/67 (47.2%)	90/93 (96.7%)	31/67 (46.2%)
Rome	48/52 (92.3%)	52/52 (100%)	52/52 (100%)	30/52 (57.5%)	48/52 (92.3%)	27/52 (42.3%)
Total	258/267 (96.6%)	266/267 (99.6%)	267/267 (100%)	148/239 (61.9%)	222/265 (83.7%)	63/239 (26.4%)

[a] Undiluted antigen was used in screening. Negative sera were retested with diluted antigen

[b] Sera positive in IEOP and AGDP tests, but negative in the CF test

[c] Numerator = number positive; denominator = number tested

Frame A-6 shows the appearance of the IEOP test. Frames A-7 and A-8 summarize typical results of the IEOP test in comparison with the complement fixation (CF) and AGDP test. An antibody titer of 1:1024 was attained (Table I). This compared favorably to an antibody titer of 1:512 in the CF test (Table II) and 1:256 in the AGDP test (Table III). Using sera from known cases of ASF, the IEOP test revealed a higher number of positives than the others (Table IV).*

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*Data in frames are reproduced with the kind permission of the editor of the CANADIAN JOURNAL OF COMPARATIVE MEDICINE, and also Doctors I. C. Pan, C. J. De Boer and W. R. Hess of PIADL.

ANTIGEN REQUIRED FOR IEOP AND AGDP TESTS

It is necessary to have antigen of good quality and high titer for these tests. Laboratories in the USA other than the Plum Island Animal Disease Laboratory (PIADL) are not allowed to possess or make ASF viral antigen. In the USA these tests are performed at PIADL.

Brief but detailed protocols for preparation of the necessary antigen and performing these tests are included for the benefit of laboratories and personnel where the above restrictions do not apply. Laboratory technicians who have had experience in CC and in serologic tests should be able to prepare the antigen and carry out the tests.

Directors of laboratories who wish to have personnel trained in the performance of these tests are invited to write to the Director, USDA, SEA, Plum Island Animal Disease Center, P. O. Box 848, Greenport, New York 11944, USA.

Preparation of Antigen For the IEOP and AGDP Tests

Antigen may be prepared by using a stable line of monkey kidney cells, such as the MS or Vero lines. The MS line of cells was obtained from the Razi Institute of Tehran, Iran and originally came from the National Institute of Animal Diseases in Tokyo, Japan. The Vero line of African green monkey kidney cells was obtained from Dr. A. J. Kniazeff, Naval Biological Laboratory, Berkeley, California in 1967 and has undergone more than 220 passages since then. The monolayer CC are prepared in Povitsky bottles (Corning Glass Works, Corning, N. Y.) (Pan _et_ _al_., 1972) and the Vero cells in Baxter bottles (Baxter Laboratories, Morton Grove, Illinois). Drums of Baxter bottles on a roller mill are shown in Frame B-6. The recovery

of ASF antigen from the Vero line in Baxter bottles is better than from MS cells in Povitsky bottles. A roller mill is required to make monolayers in Baxter bottles. Other types of roller mills with large bottles may also be used. Both cell lines can also be cultured in Povitsky bottles, plastic CC containers, or in prescription bottles as stationary cultures; amounts of antigen produced by these means may be adequate.

However, since the roller mill method employing Baxter bottles is highly productive and is now employed at the Plum Island Animal Disease Laboratory, it will be described in detail.

1. Preparation of Growth Medium for Vero Cells

1.1 A sterile 20 liter (L) carboy (large glass bottle or container) is filled with 10 L of distilled (D) H_2O.

1.2 Prepackaged CC nutrients in powder form such as that supplied by the Grand Island Biological Company, 3175 Staley Road, Grand Island, N. Y. 14072 may be used. Earle's lactalbumin hydrolysate (ELH), Catalog No. M-11, with Earle's salts, but without sodium bicarbonate is added at the rate of 13.6 gm per L. The basic formula for ELH is given in Appendix A.

1.3 Serum: 800 ml (8%) bovine serum and 200 ml (2%) of fetal calf serum are first filtered through cotton and then added.

1.4 Sodium bicarbonate ($NaHCO_3$): 22 gm are weighed and added with shaking.

1.5 Vitamins: Difco Laboratories (Detroit, Michigan, USA) TC Vitamins, Eagle, Dried, No. 5879-24 are added (1 gm vial). The basic formula for the vitamins is given in Appendix B.

1.6 Antibiotics: Sodium penicillin G, N.F. No. 8249, the Upjohn Company, Kalamazoo, Michigan 49001, USA: Dihydrostreptomycin sulfate B grade, No. 3021, Calbiochem, Los Angeles, Calif., USA and Mycostatin Sterile Powder (Nystatin, E. R. Squibb and Sons, New York, USA) are added as follows:

Penicillin: To a vial containing a million units of lyophilized sodium penicillin are added 10 ml of D H_2O; 8 ml of this per batch are added to give a final concentration of 100 units per ml.

Streptomycin: To a 5 gm vial of dihydrostreptomycin sulfate are added 20 ml of D H_2O; 4 ml of this per batch are added to give a final concentration of 100 micrograms per ml.

Mycostatin: To a vial containing 500,000 units, 10 ml of D H_2O are added; 1 ml is used per batch to give a final concentration of 50 units per ml.

1.7 Maintenance medium for seed virus is prepared with 2% fetal bovine serum. (The basic medium, minus serum, may be used as a diluent for the purification of virus.)

1.8 This work should be carried out in hoods, cubicles or other areas free of dust and aerosol contaminants.

2. Filtration: The medium is filtered through a double pass Hormann[*] filter containing two clarifying pads, followed by filtration through a 4.5 micron and a 2.2 micron Millipore filter in series under 9 lbs. nitrogen pressure. The fluid passes into 10 liter presterilized aspirator bottles (each containing a magnetic stirring bar) with glass filling bells attached.

2.1 All components are presterilized by autoclaving; hoses and connections are flamed with a portable Bunsen burner at time of assembly.

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[*] F. R. Hormann Co., Inc., P. O. Box 229, Milldale, Conn. 06467.

2.2 Frame B-6 shows drums of Baxter bottles on roller mills in a walk-in 37°C incubator. The Vero line of monkey kidney cells is grown in confluent monolayers in these bottles and used to propagate ASF virus for use as antigen in the IEOP and AGDP tests, as well as for other research purposes.

3. Preparation of Vero Cell Cultures

3.1 A saline, trypsin, versene (STV) solution is used for removal of cells from bottles. The formula for the solution is given in Appendix C.

3.2 Trypsinization of Seed Cells: Thirty ml of STV (in 1 L aspirator bottle with filling bell attached) prewarmed to 37°C is added to each Vero cell culture in a Baxter bottle. When the cells have detached, the suspension is poured into a 2 L aspirator bottle containing 1 L of growth medium as previously described.

3.2 continued

Details of the method are given: The Baxter bottles are removed from the incubator and taken to a cubicle. All manipulations are carried out with aseptic precautions, using a Bunsen burner to flame stoppers, mouths of containers, etc. Spent medium is poured off and the STV solution is dispensed with a filling bell. After this operation, either the entire drum or separate bottles are rotated to permit the STV solution to reach all cells equally.

From time to time the cells are inspected visually to observe the results of trypsinization and the bottles shaken vigorously to remove the cells.

3.3 Terminating the Trypsinization: Chilled ELH medium with 8% bovine and 2% fetal calf serum is placed on a magnetic stirrer. The fluid and trypsinized cells are then added to this from each Baxter bottle by decanting. Presence of serum will terminate the action of the STV.

3.4 Preparation of the New Culture: Five hundred ml of the decanted mixture containing the trypsinized cells (3.3) is added to 7½ L of growth fluid previously filtered into the 10 L aspirator bottles. The solution is mixed well on a magnetic stirrer and dispensed in 200 ml amounts into sterile empty Baxter bottles held in roller drums. The drums are loaded onto the roller mill at 37°C and allowed to rotate at 10 revolutions per hour for 4 days to achieve confluency.

3.5 Terminating the Trypsinization:

Chilled MEM medium with 5% bovine and 2% fetal calf serum is placed on a magnetic stirrer. The fluid and trypsinized cells are then added to this, and each flask [illegible] [illegible] [illegible] [illegible] [illegible] of serum [illegible] terminate the action of the [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] The hundred ml of the [illegible] mixture containing the trypsinized [illegible] (0.2%) is added to 7½ l of growth [illegible] and [illegible] filtered into the 10 l aspirator bottles. The solution is mixed well on a magnetic stirrer and dispensed [illegible] 250 ml [illegible] into sterile empty Baxter bottles held in [illegible] [illegible]. The [illegible] are [illegible] into [illegible] roller [illegible] at 37°C and allowed to rotate at 15 revolutions per hour [illegible] [illegible] days to [illegible] confluency.

4. Infection of Cell Cultures

4.1 Drums of Baxter bottles with confluent cell cultures are removed from the roller mill to a cubicle. Growth medium is decanted and 10 ml of seed virus at high titer (about $10^{7.8}$ HAd per ml) added by means of an automatic syringe and 1 L asperator bottle on a magnetic stirrer.

The drums of Baxter bottles with the infected cells are returned to the incubator for a 4-hour adsorption period. The roller mill is operated at 60 rotations per hour.

4.2 Maintenance medium added. After adsorption of the virus, each bottle receives 10 ml of fresh medium with serum.

4.3 Variations in procedure when CC are used for other types of virus production: (these procedures are not used for antigen production)

-- for stock virus production each bottle receives 50 ml of medium containing 2% fetal calf serum.

-- for virus purification, the cells are washed with 30 ml of phosphate buffered solution (PBS[1]) by gentle rotation. The PBS[1] is poured off and 10 ml of maintenance fluid are added. The formula for PBS[1] is given in Appendix D.

5. Harvesting the ASF Virus

5.1 The harvesting method is simple For the usual IEOP and AGDP antigen or for viral seed material, the cells are harvested with the fluid when the cells show cytopathic effect (CPE) and are sloughing from the walls of the bottles. This usually occurs between 48 and 72 hours after inoculation.

5.2 Where purer viral antigen or virus is required, the fluid portion only in the Baxter bottles is harvested at 24 hours postinoculation.

5.3 Harvesting Technique: Each bottle is shaken vigorously to remove remaining cells and the cell-fluid mixture decanted. The mixture is then centrifuged at 450 X G in the cold (6°C) for 30 minutes. A centrifuge* with an 8 inch radius measured to 1/2 the depth of fluid in the centrifuge tube was used at a speed of 2,000 revolutions per minute (r.p.m.) at 6°C. (Appendix E has a graph for converting relative centrifugal force /r.c.f. or G7 to r.p.m. or vice versa.)

The pellet, which contains the cellular debris and most of the virus, is saved for further treatment and use as IEOP and AGDP antigen. (The fluid portion also contains some virus and may be retained, but it is not used for antigen.)

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*Model PRJ of the International Equipment Company, Needham, Mass., USA.


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SONIFIER CEL
MODEL
HEAT SYSTEMS—ULTR

6. Completion of Antigen Preparation

6.1 The packed cell debris is resuspended in 2 volumes of PBS and sonicated for 2 minutes (C1,C2). This is followed by ultracentrifugation in a Spinco* 40 rotor for 60 minutes at 35,000 r.p.m. (C3,C4). The supernate is carefully removed from the ultracentrifuge tube by syringe and canula (C5,C6) and transferred to another container for storage at -70°C. Sodium azide (Na_3N) is added to give a final concentration of 0.1%.

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*Beckman Instruments, Inc., Spinco Division, Route 22 at Summit Boulevard, Mountainside, New Jersey 07091.

THIS COMPLETES THE FIRST STEP -- THAT OF ANTIGEN PREPARATION -- FOR EITHER THE IEOP OR AGDP TESTS.

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NEXT, PROTOCOLS FOR PERFORMING THE IEOP TESTS WILL BE GIVEN.

7. Performing the IEOP Test

7.1 Equipment

Any suitable immunoelectrophoresis apparatus with a power pack developing 500 volts may be used. The tests described here were carried out with a Gelman Power Supply No. 38206 (B-8) and accessories for immunoelectrophoresis (Basic Outfit No. 51841 and Screening Kit No. 51482 are typical).

7.2 Buffer Solution

An electrophoresis (barbital) buffer solution of 0.1 ionic strength, pH 8.6 is prepared according to the formula given in Appendix F.

7.3 Agar Gel

An agarose or Noble (Difco, Detroit, Michigan, USA) agar is prepared for coating the slides according to the formula given in Appendix F.

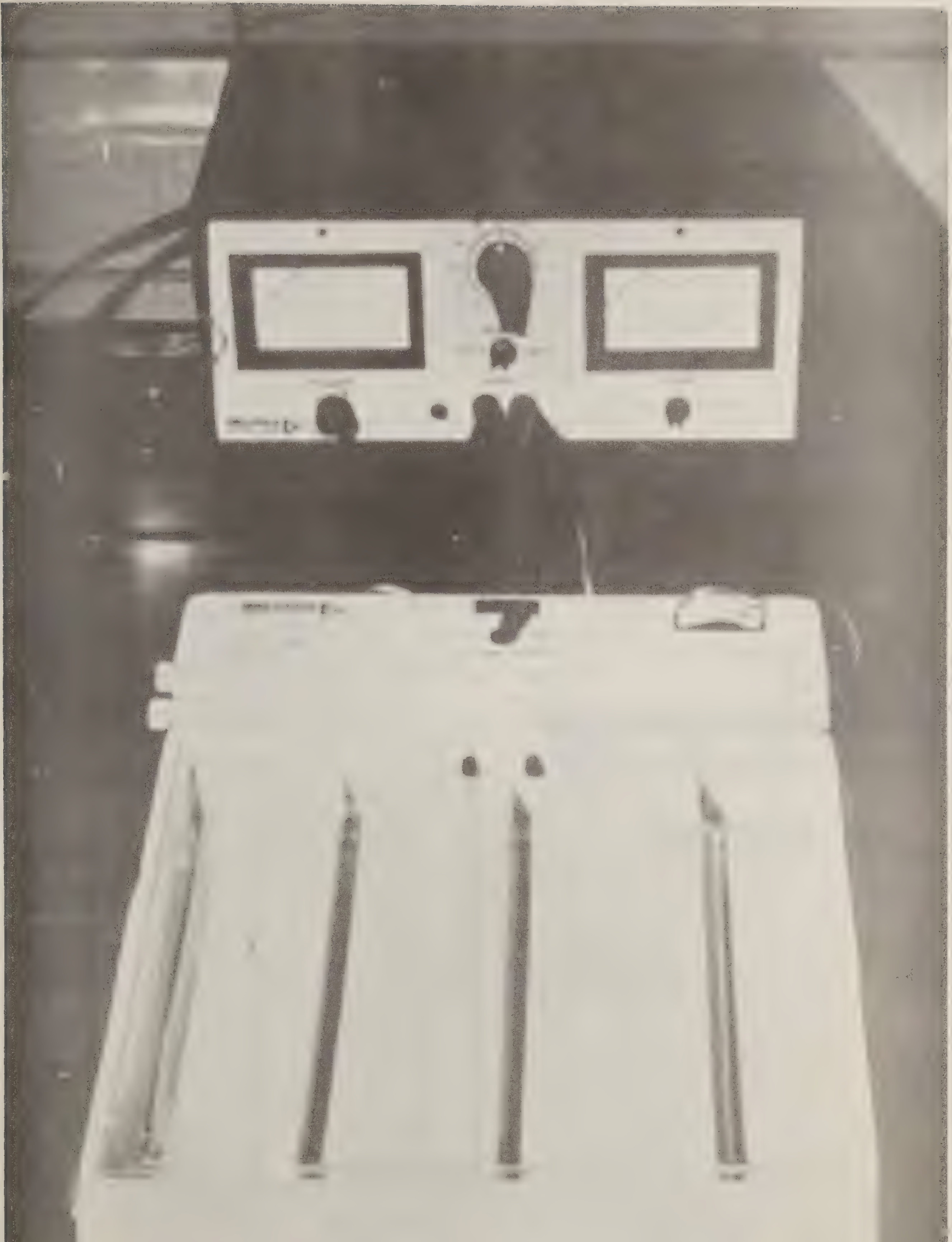
7.4 COMMENT ON FRAMES C-7, C-8 and C-9

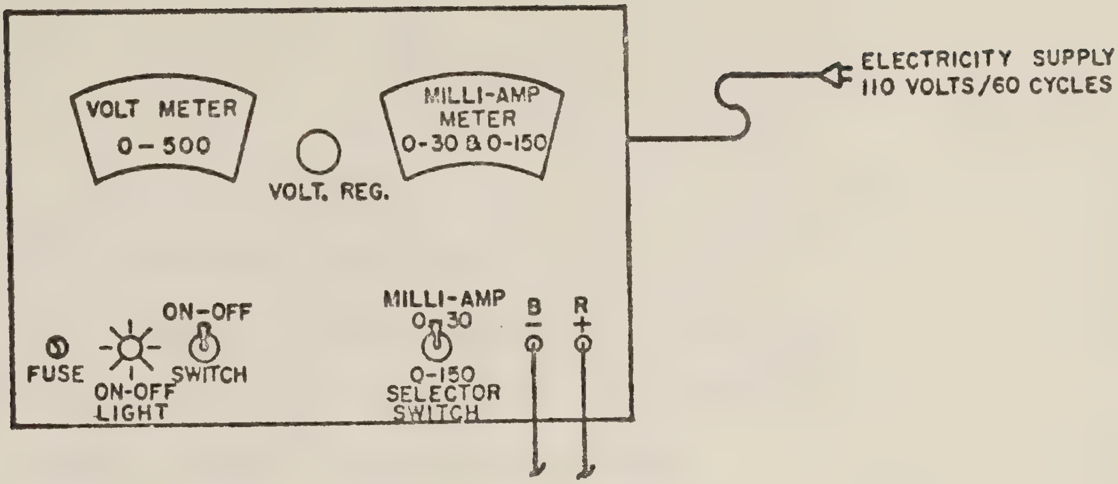
FRAME C-11 shows the chief mechanical devices used in the IEOP test. To the left rear is the POWER CONVERTER or POWER SUPPLY. In front of it is a LEVELING TABLE SET with a SPIRIT LEVEL in the center of the table. On the right front is an ELECTROPHORESIS CHAMBER.

FRAME C-12 shows another model of the power converter and electrophoresis chamber.

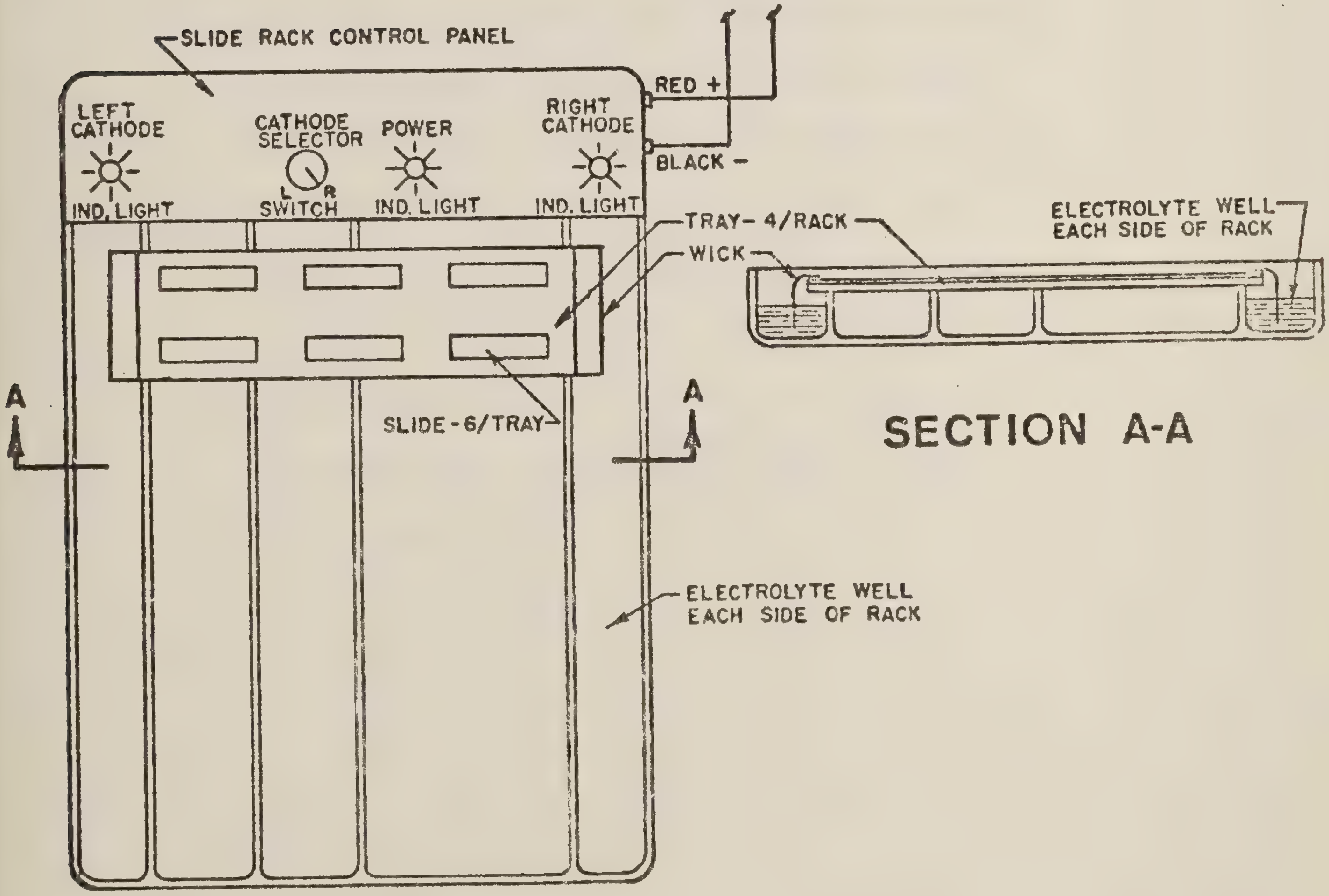
FRAME D-1 is a highly diagrammatic sketch of the power converter and electrophoresis chamber to aid in identification of the components and use of the units.


= 350 volts
= 360 volts
= 300 volts
POWER
VOLTAGE
RANGE
OUTPUT
Miscellaneous
Racks
Staining trays



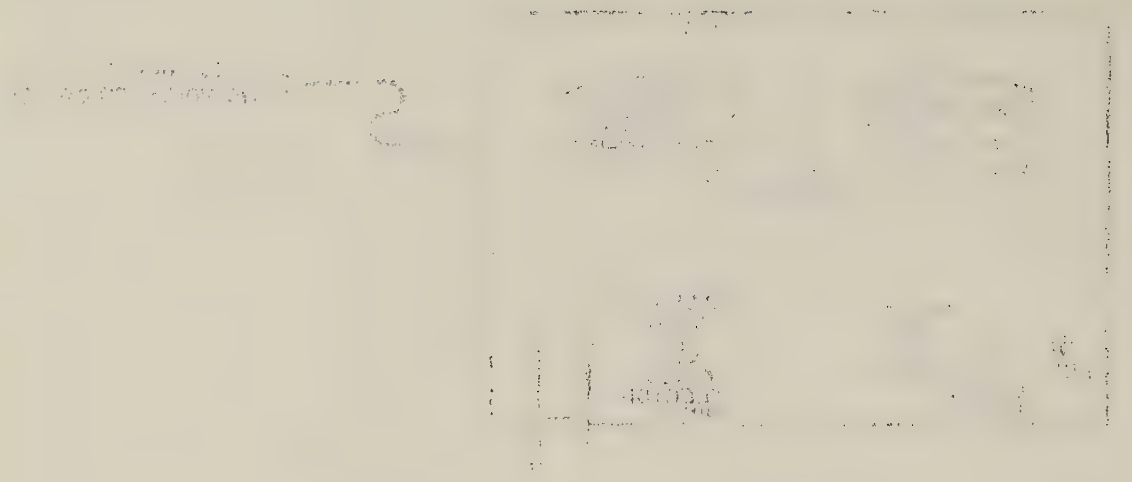


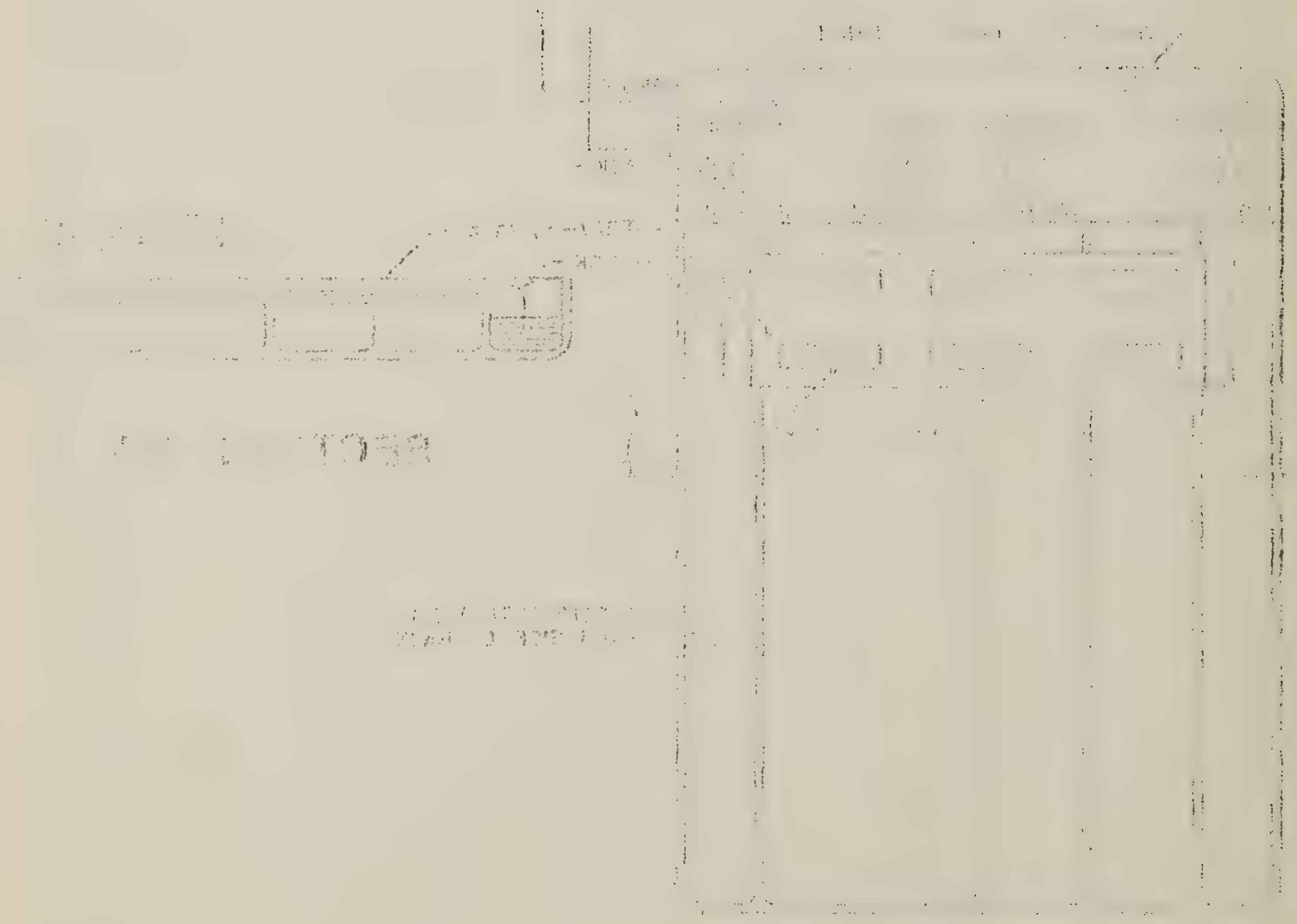
POWER CONVERTER



SECTION A-A

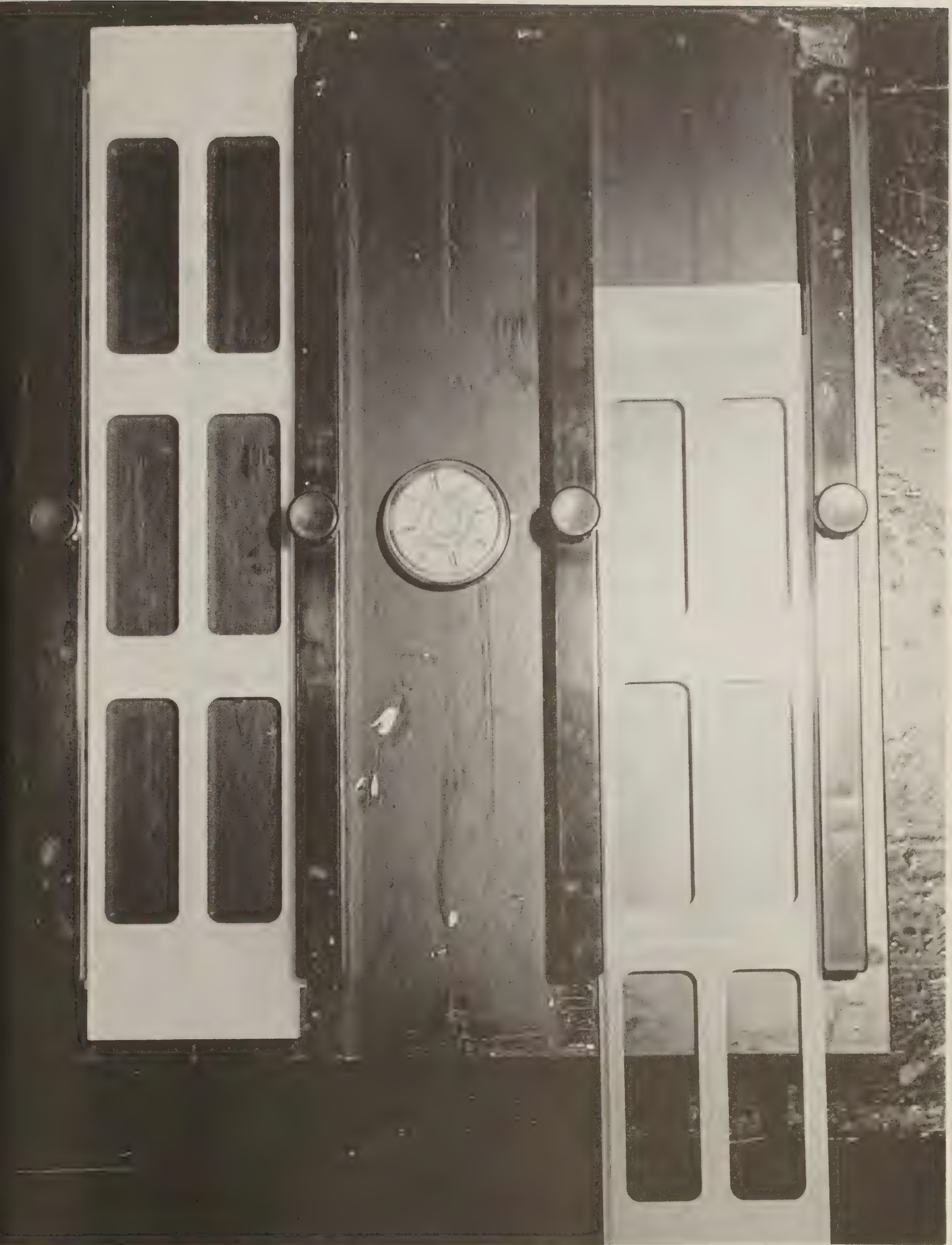
SLIDE RACK



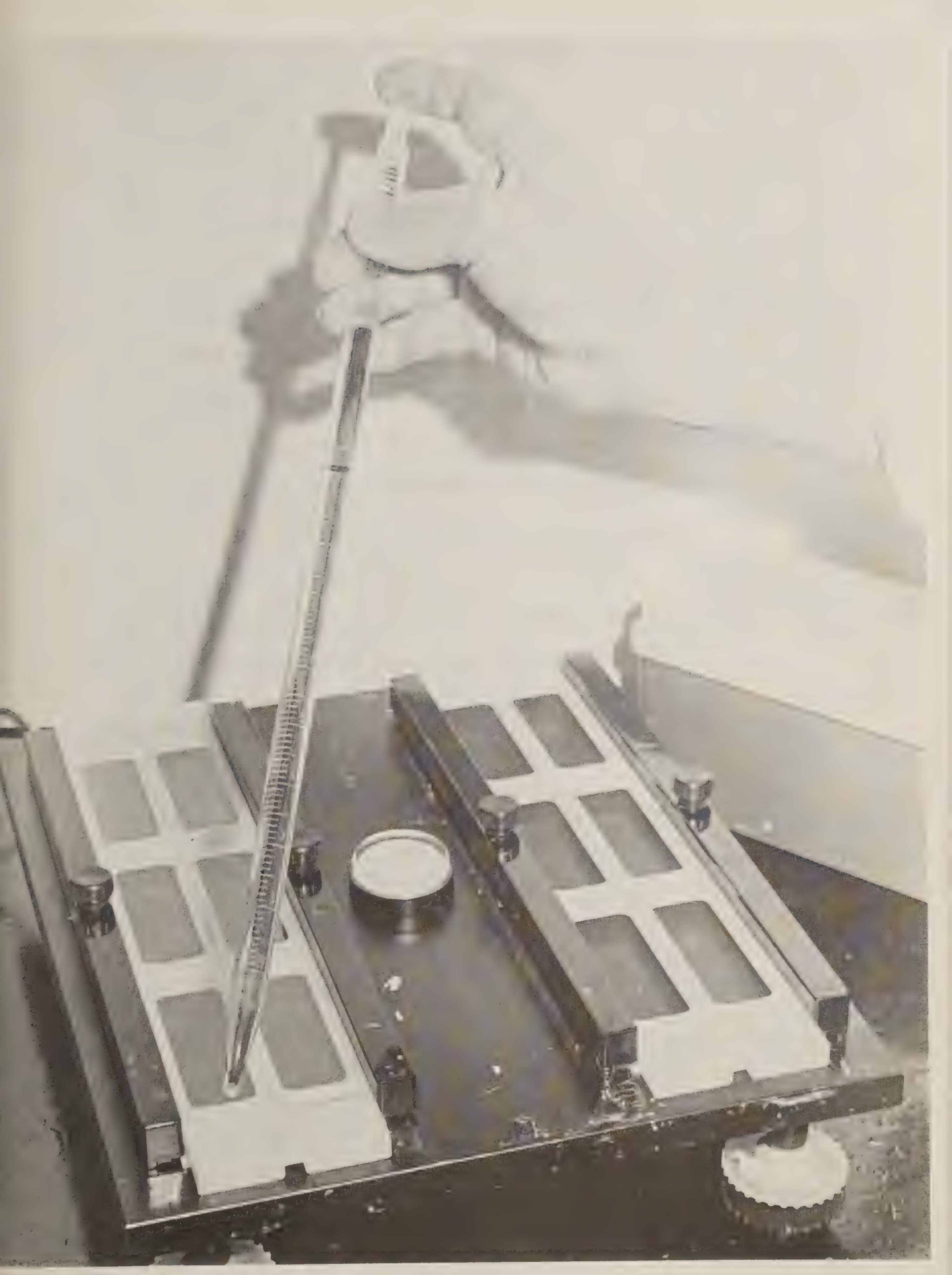


8. Preparing the Slides

With the Gelman apparatus shown, in Frames C-11 and C-12, a maximum of 192 serum samples may be tested simultaneously. Six ordinary glass slides (about 2.5 cm by 7.5 cm) each are held in a frame divided into two sections with three slides placed end to end in each section. Two movable front screws of the leveling table are adjusted until the bubble of the removable spirit level is centered. The frame is then firmly clamped on the leveling table. This assures even thickness of agar gel on the glass slides.

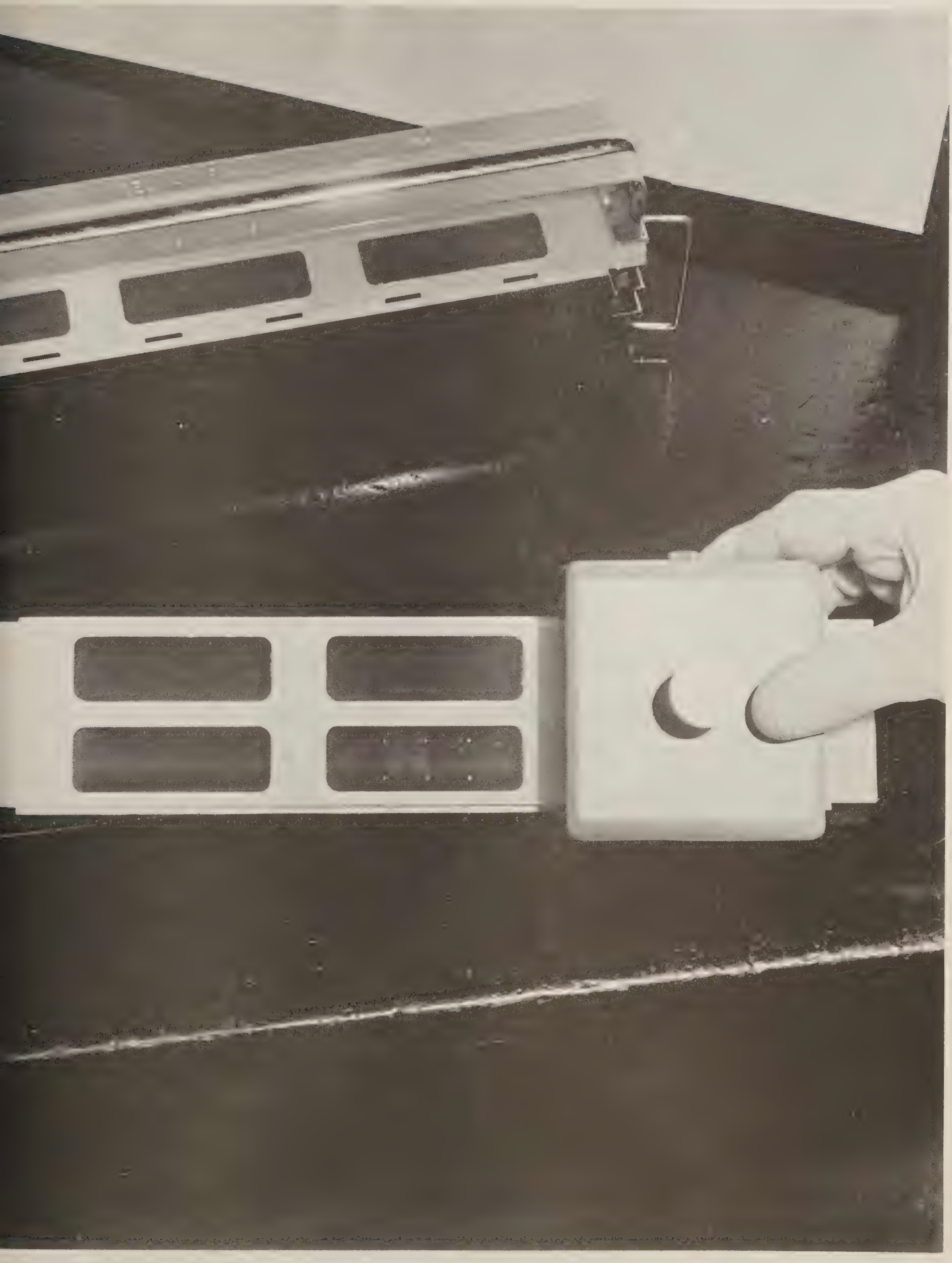


A small amount of melted agar gel is introduced at the junctures of the slides and allowed to spread by capillary action between the slides and the frame. The agar gel is allowed to solidify and form a seal which will prevent leakage when the agar gel is applied to the surface. Ten ml of melted agar gel are then pipetted on each section of three slides to form a continuous layer of uniform thickness covering the three slides. This is repeated on the other three slides of the frame. The agar gel is allowed to solidify for 30 minutes before use.



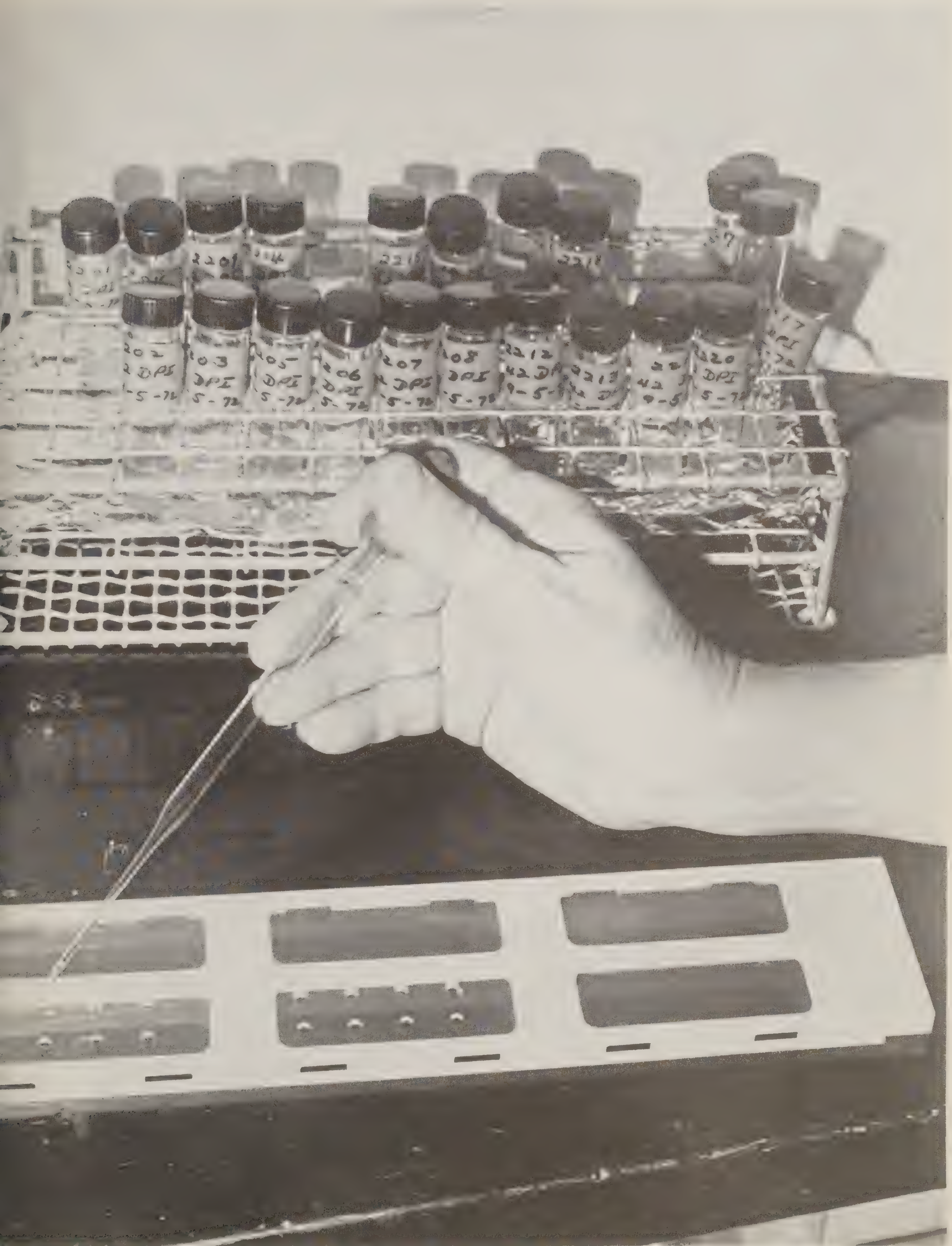
9. Preparing the Wells

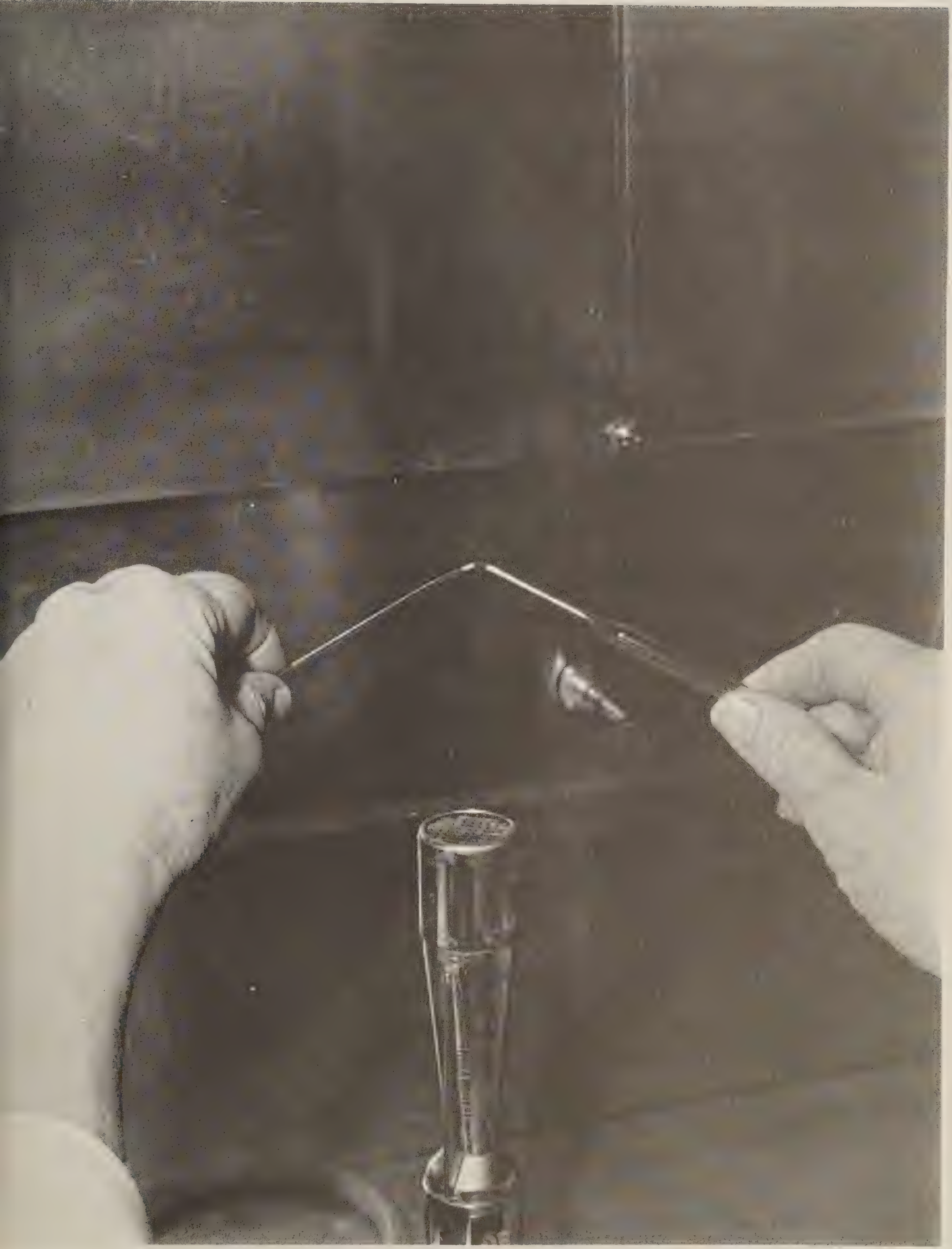
Although wells may be cut by cork borers, using a pattern, greater accuracy is obtained when a commercially available pattern cutter is employed. The wells are 3 mm in diameter and spaced at 10 mm between edges of wells. The gel plugs are removed by a 12 gauge canula, or Pasteur pipette connected with tubing to a vacuum source. A trap to prevent the agar gel plus from entering the vacuum source hose is made by interposing a flask between the canula hose and the vacuum source line.



10. Preparing Special Filling Pipettes

A special pipette is used to insure greater speed and accuracy in filling the wells. Ordinary Pasteur pipettes are rapidly converted to angle-tip micropipettes. The tip of the regular pipette is heated above a Bunsen burner, pulled out to about 6 inches and the glass bent to an approximate 45° angle. It is raised to cool the glass and the tip bent inward until the microtube portion breaks; usually the bent portion remaining is about 2 cm in length.

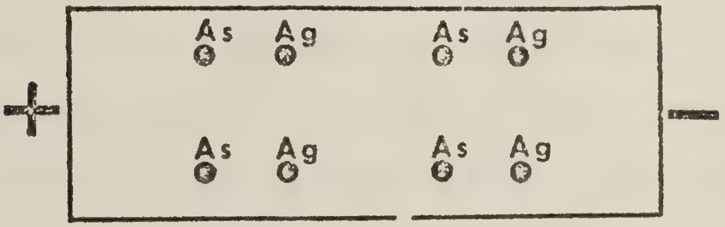

2201
2214
2215
2218
202 2 DPI
203 DPI 5-72
205 DPI 5-72
206 DPI 5-72
207 2 DPI 5-72
208 DPI 5-72
2212 42 DPI 9-5-
2213
2220 DPI 5-72



11. Filling the Wells

The wells may be filled accurately and rapidly with the special micropipette and a bulb (E-1). Note that for extra steadiness, the hand may be rested on the bench surface. A separate pipette must be used for each sample. (The antigen wells may all be filled with one pipette.)

Suspect ASF antiserum is placed in one of a pair of wells nearest the anode (+). The antigen is placed in the other well of the pair nearest the cathode (-).



11.1 COMMENT ON FRAME E-4

The Gelman power supply and electrophoresis chamber are shown in the photograph. One immuno-frame is in place within the chamber, which will hold four. The manufacturer recommends that not more than three frames be used at a time in some chambers, such as the one shown in Frame C-12. Comparison with the drawing in the next frame after the photograph will make components and their use clear. (Please note that the drawing is somewhat simplified and diagrammatic; for instance, the wells on each side are more extensive than shown.) The placement of the double wicks is best shown in the drawing. Only the top of the wicks, white bands across each end of the immuno-frame, can be seen in the photograph.


=450 volts
=250 volts
=360 volts
meter
=300 volts
VOLTAGE
RANGE
OUTPUT
−
+
OFF
GELMAN
INSTRUMENT
COMPANY
CATHODE
SELECTOR
POWER
TO
ELECTRODES
GELMAN
INSTRUMENT
COMPANY

12. Electrophoresis

The bridges of the electrophoresis chamber should be adjusted to the second position from the outer edges of the chamber. Four frames of 6 slides each may be placed across the support bridges, although only one is shown in the illustration. Wicks (for conduction of current through the gel) of cellulose acetate are saturated in the electrophoresis buffer solution described. One end of a wick is placed on each end of the agar and the other end of the wick allowed to rest in the buffer solution of the chamber. Double wicking or placing two thicknesses of wick is recommended.

The usual voltage setting for electrophoresis is 19 volts per cm or 450 volts when using the Gelman apparatus. With some chambers this voltage must be reduced when three immuno-frames are used. The test can usually be performed satisfactorily with voltages between 300 and 450. The most satisfactory length of time at PIADL has been 30 minutes. To determine the optimal duration for particular apparatus and reagents, test runs of from 15 to 60 minutes may be made.

The life of a buffer solution may be extended by alternating the anode and cathode. With the Gelman apparatus this is done simply by the setting of a knob. With other types of apparatus lacking such a device, the chamber might be reversed. When the direction of electric current is thus changed, the wells must be filled in the opposite manner, making sure that the serum is on the side nearest the anode and the antigen on the side nearest the cathode. By reversing the current flow in this manner the buffer solution may be used for 10 or more electrophoretic runs. When a total of 4 frames are used each time, the longevity of the buffer is increased proportionately. For example, if only 1 frame were used for each run, the life of the buffer would be increased fourfold.

13. Final Checks Before Electrophoresis

The antisera must be in the wells nearest the anode (+) and the antigen nearest the cathode (-). The direction of electric current flow is determined to be in the appropriate direction. No chamber components should be touched while the current is flowing as there is danger of electric shock. (The Gelman apparatus is equipped with a safety feature which turns off the electric current to the chamber when the lid is lifted.)

At the end of electrophoresis, the current is shut off and the frames removed for viewing by indirect transmitted light. A viewing box is convenient; the one shown has an adjustable mirror. A precipitin line or lines between the two wells indicates a positive test.


SORVALL
SUPERSPEED
RC2-B


Lisbon
Ag
Rome
Ag
+
−

For a permenent record, the wet agar gel slide may be photographed. Slides may also be preserved by washing for a 48-hour-period each time, in two changes of 2% NaCl solution, followed by a 1-hour rinse in D H_2O. Slides are then air dried and stained with amido (Amido Black 10B dye, Bayerwerke, Leverkusen, Germany) or buffalo (Buffalo Black, NBR, Allied Chemicals, New York) black. Six gm of one of these dyes are dissolved in a mixture of methyl alcohol (450 ml), D H_2O (450 ml) and glacial acetic acid (100 ml); then filtered through two layers of gauze before use. The decolorizing solution is made by the same formula with omission of the dye. Decolorizing is carried out until the agar gel background is clear and the lines well delineated. Slides are subsequently numbered directly on the

dried agar film with India ink and stored in a dry place. This slide can be used as a "negative" in a photographic enlarger and the image printed on photographic paper. The negative image resembles the lines as viewed by indirect light before staining; these reproductions are suitable for record or for publication.

This completes the description of the IEOP test.

* * * * *

The next section of this microfiche gives protocols for performing the AGDP test. This test is similar to the IEOP test, but is much simpler and requires little equipment and no special laboratory items. The same antigen is used for both tests. The next frame summarizes the basic steps in the AGDP test.

The Agar Gel Diffusion Precipitation Test

The AGDP test is useful as a research and diagnostic tool. If adequate amounts of either antigen or antisera are available in the fluids or tissues of the suspect animal, the test may be performed in from 18 to 48 hours. The technique is simple and requires no elaborate equipment or costly supplies. If a known ASF antisera is available, the test may be performed by placing fluids or tissues of an animal which died of suspected ASF in wells opposite the antisera. Viral antigen has been demonstrated from the blood, spleen, liver, and kidney of such animals.

If a known ASF antigen, such as that made for the IEOP test, is available, sera or other fluids from suspect animals may be used to detect specific antibodies.

The same method of washing slides in saline and D H_2O given in the previous section must always be used, as putrid or macerated tissue may result in non-specific lines when AGDP plates are viewed before adequate washing.

The agar gel and buffer solution used are identical to those described in detail for the IEOP test. The method of preparing slides is also the same, except that individual slides or Petri dishes are poured without using the immunoframes. Three ml of melted agar per slide or 15 ml of melted agar per 8.5 cm Petri dish are used.

Wells may be cut in a variety of patterns so that antigen and antisera are placed in opposite wells. Frequently a circular pattern is used with the chief reagent placed in the central well and test sera, tissue or fluids in the peripheral wells. Thus the central well is filled with antigen when testing for the presence of antibodies in the peripheral wells. Frame F-7 is a photograph of an AGDP test; in this case a template was

used to cut the central well and 6 peripheral wells at one time.

The well cutting template consisted of a metal disc to which six 4 mm sharpened metal tubes were affixed in a circle around a central tube of the same diameter. The approximate distance from the outer edge of the central well to the near edge of each peripheral well was 5 mm.

COMMENT ON THE NEXT FRAME:

This is a photograph of a typical AGDP test. Wells were filled as follows:

Center Well: ASFV Antigen

Peripheral Wells 1, 3 and 5: Normal Swine Serum

Peripheral Wells 2, 4 and 6: Sera from swine which recovered from ASF.

As in the IEOP test, the agar must be washed properly in saline to remove nonspecific lines. By placing antisera in the central well it is possible to test for the presence of antigen by placing bits of tissue or body fluids from suspect animals in the outer wells. Dr. W. R. Hess of PIADL states that in case of tissues containing ASF virus, it is probable that only one line may appear. For a more complete discussion of the IEOP and AGDP tests for ASF, reference should be made to Pan, De Boer and Hess, 1972 (full reference given in Frames A-1 and A-2).

This completes the description of the AGDP test.

* * * * *

The next section (Fiche 2) comprises a brief description of the FA test. This technique is quite different from the two previously described. Its basic purpose is to demonstrate ASF virus or viral antigen in tissues. The first frame in the second fiche summarizes the basic steps in performing this test.

Appendix A

The formula for Earle's salts in the ELH medium is:

Component	mg/L
NaCl	8,000.0
KCl	400.0
NaH_2PO_4	60.0
$CaCl_2$ (anhydrous)	140.0
$MgCl_2 \cdot 6\ H_2O$	100.0
$MgSO_4 \cdot 7\ H_2O$	100.0
KH_2PO_4 (anhydrous)	60.0
Glucose	1,000.0
Lactalbumin Hydrolysate	6,500.0
Phenol red	10.0
$NaHCO_3$	350.0

Appendix B

The formula for the amount of added vitamins is:

Vitamin	Amount
D Biotin	0.24 mg
Folic acid	0.44 mg
Niacinamide	0.12 mg
Calcium pantothenic	0.24 mg
Pyridoxal hydrochloride	0.20 mg
Thiaminde hydrochloride	0.34 mg
Riboflavin	0.04 mg
Choline chloride	0.14 mg

Appendix C

Formula for Saline, Trypsin, Versene (STV) Solution

Component	Amount
NaCl	8.0 gm
KCl	0.4 gm
Dextrose	1.0 gm
$NaHCO_3$	0.58 gm
Trypsin (Difco 1:250)	0.5 gm
Versene (EPTA) (Disodium salt 1:250)	0.2 gm
D H_2O to make	1,000 ml

Filter and store below -20°C; do not refreeze and use again.

Appendix D

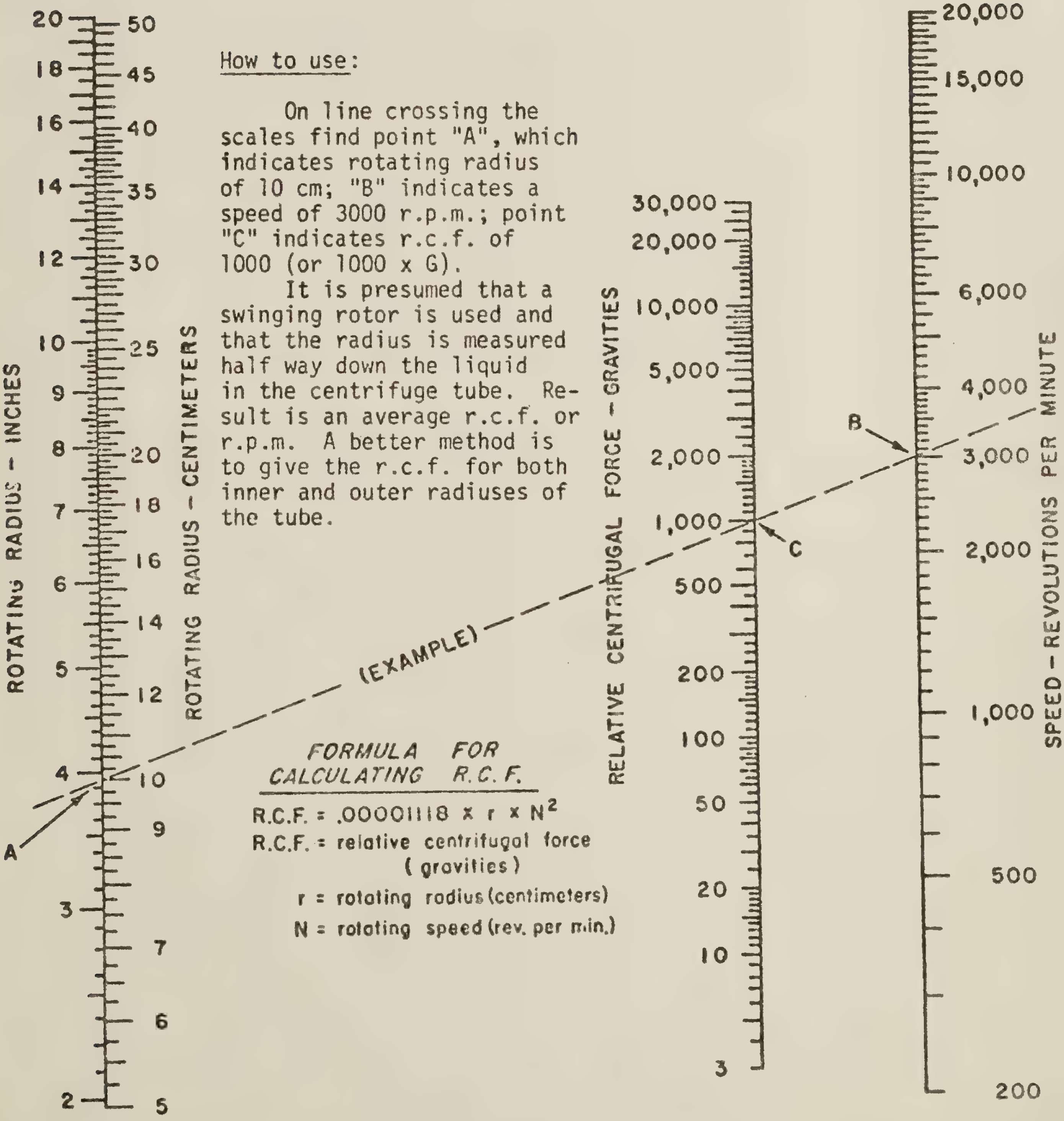
Formula,

Phosphate buffered solution (PBS[1]) for purification of ASF virus

NaCl	8.0	gm
KCl	0.2	gm
$Na_2HPO_4 \cdot 7\ H_2O$	2.172	gm
KN_2PO_4	0.2	gm
D H_2O	1,000	ml

Appendix E

LABORATORY AID FOR OBTAINING RELATIVE CENTRIFUGAL FORCE (G) or REVOLUTIONS PER MINUTE (RPM)



How to use:

On line crossing the scales find point "A", which indicates rotating radius of 10 cm; "B" indicates a speed of 3000 r.p.m.; point "C" indicates r.c.f. of 1000 (or 1000 x G).

It is presumed that a swinging rotor is used and that the radius is measured half way down the liquid in the centrifuge tube. Result is an average r.c.f. or r.p.m. A better method is to give the r.c.f. for both inner and outer radiuses of the tube.

FORMULA FOR CALCULATING R.C.F.

$$\text{R.C.F.} = .00001118 \times r \times N^2$$

R.C.F. = relative centrifugal force (gravities)

r = rotating radius (centimeters)

N = rotating speed (rev. per min.)

Appendix F

Electrophoresis Buffer Solution:

(Barbital buffer solution, ionic strength 0.1, pH 8.6).

Sodium barbital	13.38 gm
Sodium acetate (3 H_2O)	8.83 gm
Distilled H_2O to make	1.50 liters

Adjust pH with conc. HCl to pH 8.6

Electrophoresis Agar Gel:

6 gm agarose, or 10 gm Noble (Difco, Detroit, Mich., USA) special agar

10 ml, 10% sodium azide

250 ml barbital buffer, above

750 ml distilled water

Heat to 100°C until agarose is melted for use.